Amino Acids (2002) 22: 427–432

Amino
Acids

Thin layer chromatographical detection of tyrosine produced from L-[U-^{14}C]phenylalanine by ruminal microbes

Short Communication

R. I. Khan[1], **R. Onodera**[2], and **M. R. Amin**[1]

[1]Department of Animal Science, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh, Bangladesh
[2]Laboratory of Animal Nutrition and Biochemistry, Faculty of Agriculture, Miyazaki University, Japan

Accepted December 3, 2001

Summary. Thin layer chromatographical detection of tyrosine (Tyr) synthesized from L-[U-^{14}C]phenylalanine (Phe) (1 mM) by rumen bacteria (B) and protozoa (P) collected from fistulated Japanese Goat was carried out. About 16 and 12% of the added Phe was converted to Tyr by B and P, respectively. Large amount of radioactivity in ether fractions indicated an abundant production of aromatic acids from Phe. Small amount of radioactivity found in CO_2 fractions implied an occurrence of considerable decarboxylation reaction(s) by rumen bacteria and protozoa.

Keywords: Amino acids – Tyrosine – Phenylalanine – Rumen bacteria – Protozoa

Introduction

Using the newly developed high-performance liquid chromatography (HPLC) method (Khan et al., 1998), it was reported for the first time that Tyr can be synthesized from Phe by both rumen bacteria and protozoa (Khan et al., 1999). However, many researchers did not find any Tyr after incubation of [U-^{14}C]Phe (Scott et al., 1964; Patton and Kesler, 1967) or its precursor [1-^{14}C]phenylacetic acid (PAA) (Allison, 1965) with rumen microorganisms. Kristensen (1974), on the other hand, reported that mixed rumen bacteria could produce small amount of Tyr from PAA. Amin and Onodera (1997a; 1997b) observed that an unknown compound of same retention time of Tyr and *P*-hydroxyphenylacetic acid (HPA) by HPLC (Amin et al., 1995) increased after the degradation of Phe, PAA, and also phenylpyruvic acid (PPY) by mixed rumen bacteria and protozoa, although they could not iden-

tify the unknown compound due to the limitation of the HPLC method. Thus, the results so far reported on the formation of Tyr from Phe by rumen microorganisms are very much contradictory. In order to confirm our previous result (Khan et al., 1999) and to solve the above contradictions, the present study was conducted using L-[U-^{14}C]Phe by thin layer chromatography (TLC).

Materials and methods

Suspensions (4 ml) of mixed rumen bacteria (B) and protozoa (P) prepared from goat rumen contents as described previously (Khan et al., 1999) were transferred into the main chamber of the Warburg flask contained 10 μCi (0.2 ml) of L-[U-^{14}C]Phe (Sp. Act. 460 mCi mmol^{-1}, Nycomed Amersham, Buckinghamshire HP7 9NA, England) with 0.3 ml of L-Phe and 0.5 ml of L-Tyr so that the final concentrations of Phe and Tyr were 1 and 2 mM, respectively; cold Tyr was added to minimize the degradation of L-[U-^{14}C]Tyr. To trap CO_2, 0.7 ml of 20% KOH was placed in the center well. Both suspensions were incubated at 39°C for 6 h, mixed with 1 ml of 5N H_2SO_4 and centrifuged at 27,000 $\times$ g for 30 min at 20°C. Supernatant fluid was washed with ether to separate ether-soluble fraction. The precipitates were hydrolyzed with 6 M HCl (6 ml) at 110°C for 20 h according to the previous method (Khan et al., 1999). Water-soluble fraction and hydrolyzate were desalted by percolation through Amberlite CG-120 (H form) resin and amino acids were eluted with 2N NH_4OH. Aliquotes from CO_2 fraction, ether-soluble fraction, water-soluble fraction and hydrolyzate were taken into scintillation vial for counting radioactivity.

Thin layer chromatography (TLC) of the water-soluble fractions and the hydrolyzates was carried out by using (1) *n*-BuOH : AcOH : H_2O (4 : 1 : 1) and (2) Phenol : H_2O (4 : 1) as solvents for two dimensions according to the method of Rockland and Underwood (1967). Amino acids were primarily located through the reaction with ninhydrin spray and the spots for Phe and Tyr were identified by the comparison of their Rf values with those of the respective standard ones. Radioactivity of all the spots present on the chromatograms were examined and measured by a computer with radio-analytic imaging system (Hewlett Packard Vecta, California). Autoradiographs were prepared by exposing the chromatograms (TLC) to X-ray film.

Results and discussion

Degradation of Phe and distribution of radioactivity in each fraction

The degradation rates of Phe in B and P suspensions were 50 and 34%, respectively, after 6 h incubation. These were in accordance with the results reported by Amin and Onodera (1997a), but lower than that reported by Khan et al., (1999). The differences may be due to the microbial number and the species included in suspensions (Scheifinger et al., 1976). Large amounts of radioactivity (29 and 18% in B and P, respectively) were found in the ether fractions, indicating an abundant production of aromatic acids by the degradation of Phe. It has been reported that Phe was degraded by rumen bacteria and protozoa to produce mainly PAA and considerable amounts of phenylpropionic acid (PPA), benzoic acid, and HPA (Scott et al., 1964; Patton and Kesler, 1967; Amin and Onodera, 1997a; Khan et al., 1999). In the CO_2 fractions, 9 and 5% of the total radioactivity were found in B and P, respec-

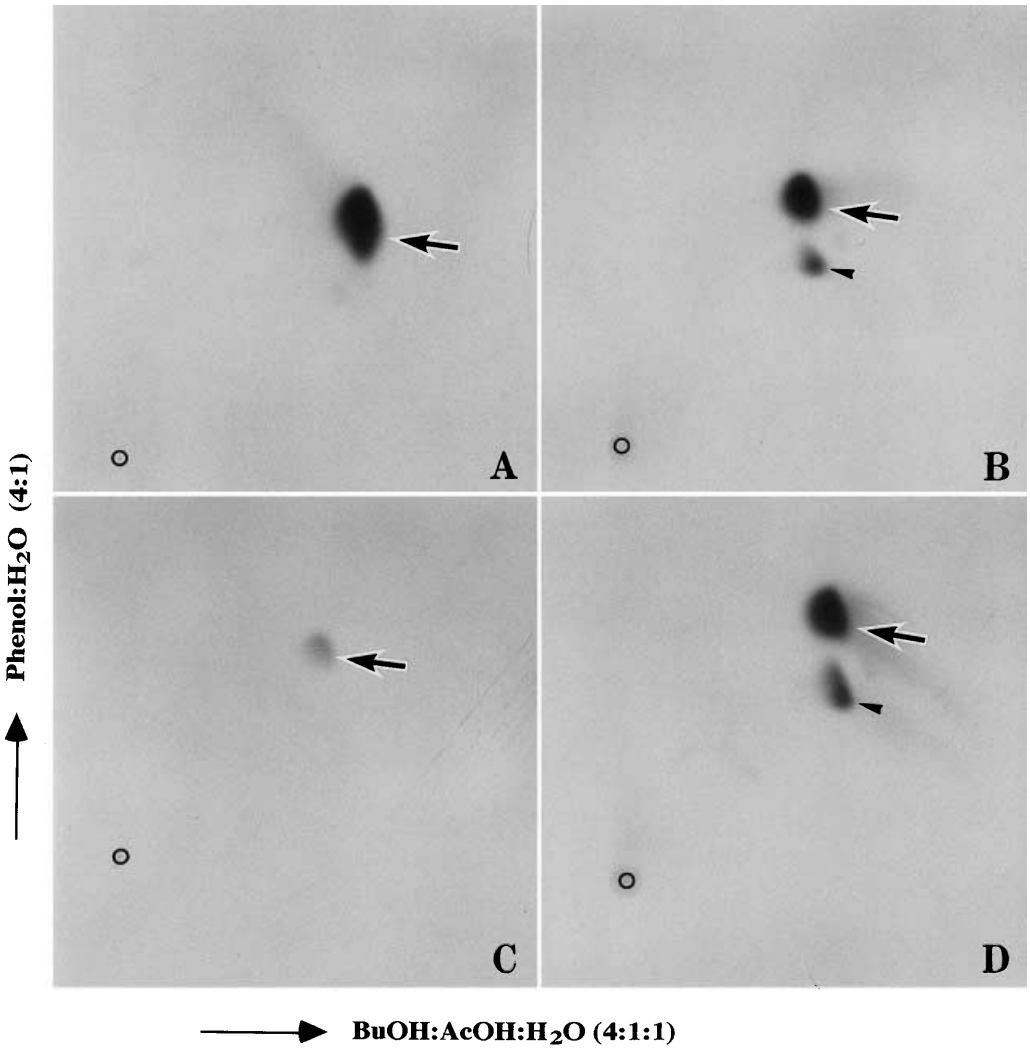

Fig. 1. Autoradiographs of TLC of amino acids contained in the water-soluble fractions (**A, B**) and hydrolyzates (**C, D**) of rumen bacterial suspensions before (**A, C**) and after 6 h incubation (**B, D**) with L-[U-^{14}C]phenyalanine. Circles, arrows & arrow heads are indicating starting points on TLC, spots of radioactive phenylalanine and tyrosine, respectively

tively. The distribution of radioactivity in the CO_2 fraction implies a considerable amounts of decarboxylation of Phe and its metabolites occurred during the period. The water-soluble fractions of B and P contained 27 and 60% of the total radioactivity, respectively, being mainly undegraded Phe and the produced Tyr (Figs. 1B and 2B). Comparatively a larger amount of radioactivity was observed in P than that in B, because the degradation rate of Phe in P is lower than that in B (Amin and Onodera, 1997a; Khan et al., 1999). Moreover, protozoa liberate endogenous amino acids into the medium from their cell protein during incubation (Onodera and Kandatsu, 1970). About 28 and 8% of the added radioactivity were found in the hydrolyzates of B and P, and

most of the radioactivity were detected in Phe and Tyr (Figs. 1D and 2D). A large amount of radioactivity was observed in B because of much incorporation of radioactive Phe and Tyr (Fig. 1D) into the bacterial cells (Broderick et al., 1991; Armstead and Ling, 1993; Ling and Armstead, 1995).

Production of Tyr from Phe

Autoradiographs of TLC of the water-soluble fractions and hydrolyzates of B and P showed a clear radioactive new spot other than a Phe spot (Figs. 1B, 1D, 2B and 2D) after 6 h incubation, while only Phe was observed before incubation (Figs. 1A, 1C, 2A and 2C). The new spot was identified as Tyr from the

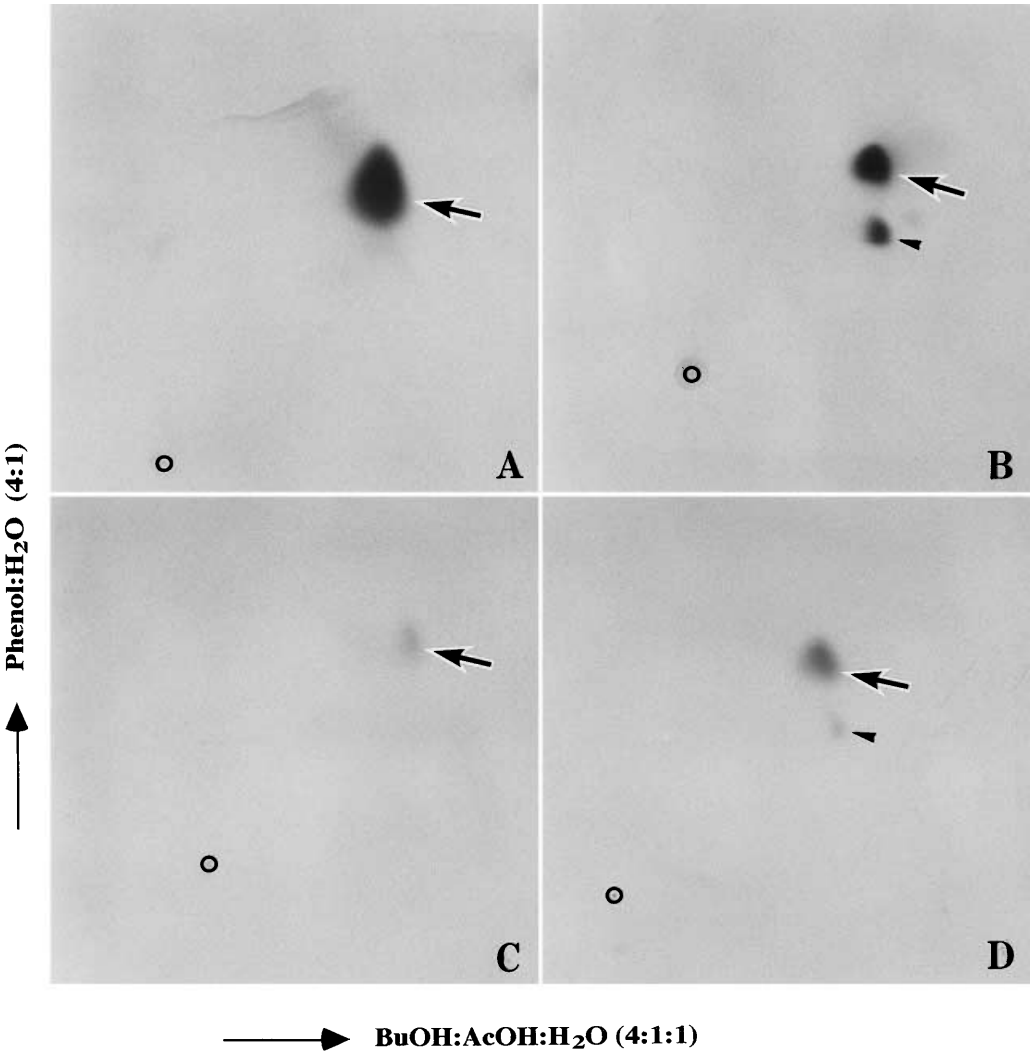

Fig. 2. Autoradiographs of TLC of amino acids of rumen protozoal suspensions. Other indications see Fig. 1

Rf value. In the water-soluble fractions about 5 and 10%, in hydrolyzates about 11 and 2%, and thus totally about 16 and 12% of the added Phe was converted to Tyr during a 6-h incubation of B and P, respectively.

Although there have been contradictory results among researchers (see Introduction) this study confirms that both rumen bacteria and protozoa can produce Tyr from Phe in an anaerobic condition, because radioactive Tyr was found on the chromatograms of water-soluble fractions and hydrolyzates of B and P suspensions after 6 h incubation of L-[U-^{14}C]Phe.

Acknowledgments

We would like to thank the Ministry of Education, Science, Sports and Culture, Japan (Monbusho) for the award of research scholarships to Rokibul Islam Khan since 1996. The present study was financially supported by the Grant-in-Aid for JSPS Fellow, M. Ruhul Amin (1998–2000), research grants from Ajinamoto Co., Inc., Tokyo, Japan and Kyowa Hakko Kogyo, Japan.

References

Allison MJ (1965) Phenylalanine biosynthesis from phenylacetic acid by anaerobic bacteria from the rumen. Biochem Biophys Res Commun 18: 30–35

Amin MR, Onodera R (1997a) In vitro metabolism of phenylalanine by ruminal bacteria, protozoa, and their mixture. J Gen Appl Microbiol 43: 1–7

Amin MR, Onodera R (1997b) Synthesis of phenylalanine and production of other related compounds from phenylpyruvic acid and phenylacetic acid by ruminal bacteria, protozoa, and their mixture in vitro. J Gen Appl Microbiol 43: 9–15

Amin MR, Tomita Y, Onodera R (1995) Rapid determination of phenylalanine and its related compounds in rumen fluids by high-performance liquid chromatography. J Chromatogr 663: 201–207

Armstead IP, Ling JR (1993) Variations in the uptake and metabolism of peptides and amino acids by mixed rumen bacteria in vitro. Appl Environ Microbiol 59: 3360–3366

Broderick GA, Wallace JR, Ørskov ER (1991) Control of rate and extent of protein degradation. Physiologocal aspects of digestion and metabolism in ruminants. In: Tsuda T, Sasaki Y, Kawashima R (eds) Academic Press, San Diego, pp 541–592

Khan RI, Amin MR, Mohammed N, Onodera R (1998) Quantitative determination of aromatic amino acids and related compounds in rumen fluid by high-performance liquid chromatography. J Chromatogr 710: 17–25

Khan RI, Onodera R, Amin MR, Mohammed N (1999) Production of tyrosine and related compounds from phenylalanine by rumen microorganisms. Amino Acids 17: 335–346

Kristensen S (1974) Ruminal biosynthesis of aromatic amino acids from arylacetic acids, glucose, shikimic acid and phenol. Br J Nutr 31: 357–365

Ling JR, Armstead IP (1995) The in vitro uptake and metabolism of peptides and amino acids by five species of rumen bacteria. J Appl Bacteriol 78: 116–124

Onodera R, Kandatsu M (1970) Amino acid and protein metabolism of rumen ciliate protozoa. IV. Metabolism of casein. Jpn J Zootech Sci 41: 307–313

Patton S, Kesler EM (1967) Presence and significance of phenyl-substituted fatty acids in bovine rumen contents. J Dairy Sci 50: 1505–1508

Rockland LB, Underwood JC (1967) Techniques in protein chemistry. In: Bally JL (ed) Elsevier, Amsterdam London, pp 1–40

Scheifinger C, Russell N, Chalupa W (1976) Degradation of amino acids by pure cultures of rumen bacteria. J Anim Sci 43: 821–827
Scott TW, Ward PFV, Dawson RMC (1964) The formation and metabolism of phenyl-substituted fatty acids in the ruminants. Biochem J 90: 12–23

Authors' address: Rokibul I. Khan, Department of Animal Science, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh, Fax: +880 91 55810, E-mail: rikhanah@bdonline.com

Received July 18, 2001